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(57) Abstract: The present invention provides methods to determine if a patient has or is at risk for developing a renal disorder. In certain embodiments, specific biomarkers are provided which may be used to determine if a patient has or is at risk for developing a renal disorder (e.g., acute renal failure).



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DESCRIPTION

BIOMARKERS FOR RENAL DISORDERS

BACKGROUND OF THE INVENTION

5 The present application claims benefit of priority to U.S. Provisional Application Serial No. 60/805,414 filed June 21, 2006, and U.S. Provisional Application No. 60/866,733 filed November 21, 2006, the entire contents of which are hereby incorporated by reference.

1. Field of the Invention

10 The present invention relates generally to the fields of molecular biology and medicine. More particularly, it concerns methods for the diagnosis, prediction and prevention of renal disorders including the rejection of transplanted kidney using certain biomarkers.

2. Description of Related Art

15 Current techniques for diagnosing, predicting or preventing renal disorders have significant limitations and risks. Diagnosis of glomerular and/or tubular kidney diseases and follow-up of the pathological process activity including the rejection of transplanted kidney are currently made respectively with renal biopsy and laboratory data (*e.g.*, serum creatinine, proteinuria and urinary sediment).

20 Renal biopsies have significant clinical risks and limitations. The renal biopsy allows appreciating the state of the lesions at a precise time point of the disease. However, clinicians commonly choose not to perform a renal biopsy because of the high risks including bleeding, a unique kidney and damage to the kidney. Not surprisingly, it is also not common to do a second biopsy in order to follow the morphological evolution of the glomerular lesions. Usual laboratory data, such as serum creatinine and urinary albumin levels, allow to some degree of analysis of the severity of renal function impairment. However, they are indeed
25 functional, not morphological parameters. Moreover, they are neither specific for each glomerular or tubular disorder nor sensitive enough to reflect the activity of the disease or the risk of kidney failure.

Therefore, the identification of easily detectable specific and sensitive glomerular and/or tubular markers could help nephrologists and other clinicians in their daily practice. There exists a need for biomarkers indicating podocyte lesion and tubular cell damage which could be used to diagnose, predict or even prevent renal pathologies.

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SUMMARY OF THE INVENTION

The present invention overcomes deficiencies in the art by providing specific biomarkers (*e.g.*, glomerular podocytes and tubular cell markers) in urine, plasma and/or serum that could allow the diagnosis and follow-up evaluation of glomerular and/or tubular kidney diseases as well as detecting acute or chronic rejection of the transplanted kidney .

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An aspect of the present invention relates to a method of assessing whether an individual has or is at risk for developing a renal disorder comprising the steps of: (a) obtaining a biological sample from the individual, (b) analyzing the sample to determine the presence, absence or amount of one or more biomarkers selected from the group consisting of the biomarkers of Table 1 and (c) assessing from said presence, absence or amount of the one or more biomarkers whether the individual has or is at risk for developing a renal disorder. The analyzing may comprise determining the presence or amount of one or more of pigment epithelium-derived factor, Attractin, FGF 11 (fibroblast growth factor), nestin, Dermcidin, EGF-containing fibulin-like matrix protein 1 (fibulin 3), myosin Va (non muscle), neuroligin-2, brain enriched hyaluronan binding protein, Ryanodine receptor 2, Ankyrin 3, serum paraoxonase/aryltransferase 1 (PON1), neuropilin-2 (NP-2), alpha2-macroglobulin or FERM domain containing protein 4A. The sample may be a blood sample, a urine sample, a kidney biopsy sample, a serum sample or a plasma sample.

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The analyzing of the sample may comprise performing one or more of an ELISA, an immunoassay, reverse transcription PCR (RT-PCR), a radioimmunoassay (RIA), an immunoradiometric assay, a fluoroimmunoassay, an immunoassay, a chemiluminescent assay, a bioluminescent assay, a gel electrophoresis, a Western blot analysis, a silver-stained gel analysis, an in situ hybridization assay, an immunohistochemistry assay, a protein biochip assay, a microfluidic chip-based immunoassay, a mass spectrometry assay, a GC/MS assay, a LC-MS/MS assay, a MALDI tof mass spectroscopy assay, SELDI tof mass spectroscopy assay, or a HPLC assay thereon. The analyzing of the sample may comprise performing an immunoassay thereon.

The renal disorder may be acute renal failure. In certain embodiments, the renal disorder is a glomerular disease, such as a minimal change disease, a focal or segmental glomerulosclerosis, a collapsing glomerulopathy, a membranous nephropathy, a membranoproliferative glomerulonephritis, a dense deposit disease, a cryoglobulinemia-associated glomerulonephritis, an IgA nephropathy, an Henoch-Schönlein disease, a postinfectious glomerulonephritis, a bacterial endocarditis, a pauci-immune crescentic glomerulonephritis, a Wegener granulomatosis, a microscopic polyangitis, a Churg-Strauss syndrome, an anti-GBM-antibody mediated glomerulonephritis, a lupus nephritis, or a chronic allograft glomerulopathy. The glomerular disease may have organized deposits. The glomerular disease may be a amyloidosis, a monoclonal immunoglobulin deposition disease, a fibrillary glomerulonephritis, or an immunotactoid glomerulopathy.

In certain embodiments, the renal disorder is a tubulo-interstitial disease, such as an ischemic tubular injury, a medication-induced tubulo-interstitial nephritis, a toxic tubulo-interstitial nephritis, an infectious tubulo-interstitial nephritis, a bacterial pyelonephritis, a viral infectious tubulo-interstitial nephritis which results from a polyomavirus infection or an HIV infection, a metabolic-induced tubulo-interstitial disease, a mixed connective disease, a cast nephropathy, a crystal nephropathy which may results from urate or oxalate or drug-induced crystals deposition, an acute cellular tubulo-interstitial allograft rejection, a tumoral infiltrative disease which result form a lymphoma or a post-transplant lymphoproliferative disease, or an obstructive disease of the kidney.

The renal disorder may be a vascular disease, such as a thrombotic microangiopathy, a nephroangiosclerosis, an atheroembolic disease, a mixed connective tissue disease, a polyarteritis nodosa, a calcineurin-inhibitor induced-vascular disease, an acute cellular vascular allograft rejection, or an acute humoral allograft rejection. The renal disorder may result from a hereditary disease (*e.g.*, Alport's syndrome, thin membrane disease, Fabry's disease, a polycystic kidney disease) or a renal disorder which results from a metabolic disease (*e.g.*, a diabetic nephropathy).

The biomarker may be present or absent in the biological sample. In certain embodiments, an increased amount of the biomarker in the biological sample relative to a control sample indicates that the individual has or is at risk of the renal disorder. In certain embodiments, wherein the presence of the biomarker in the biological sample indicates that the individual has or is at risk of the renal disorder.

Another aspect of the present invention relates to a method for identifying a candidate biomarker for a renal disorder comprising: (a) obtaining a biological sample from an individual who has the renal disorder, (b) purifying a podocyte vesicle from the biological sample (*e.g.*, urine); and (c) measuring the amount of the protein in the biological sample, wherein if the protein is present in the biological sample in an amount greater than is present in a control sample, then the protein is the candidate biomarker. In certain embodiments, the candidate biomarker is greater than about 40 kDa or greater than about 60 kDa. In certain embodiments, the candidate biomarker is a neuronal protein. The purifying may comprise immunopurification.

Another aspect of the present invention relates to a kit comprising: (a) a first antibody preparation that selectively binds immunologically to one or more biomarkers selected from the group consisting of the biomarkers of Table 1, and (b) a suitable container means thereof. In certain embodiments, the antibody selectively binds immunologically to pigment epithelium-derived factor, Attractin, FGF 11 (fibroblast growth factor), nestin, Dermcidin, EGF-containing fibulin-like matrix protein 1 (fibulin 3), myosin Va (non muscle), neuroligin-2, brain enriched hyaluronan binding protein, Ryanodine receptor 2, Ankyrin 3, serum paraoxonase/aryltransferase 1 (PON1), neuropilin-2 (NP-2), alpha2-macroglobulin or FERM domain containing protein 4A. The first antibody may be a monoclonal antibody or a polyclonal antibody. The antibody preparation may be attached to a support, such as a polystyrene plate, test tube or a dipstick. The kit may further comprise at least a second antibody preparation. The second antibody preparation may comprise a detectable label (*e.g.*, a fluorescent tag, a chemiluminescent tag or an enzyme). Said enzyme may be alkaline phosphatase or horseradish peroxidase. The kit may further comprise a substrate for said enzyme. The kit may further comprise a buffer or diluent and a suitable container means therefor. In certain embodiments, the kit comprises an ELISA assay.

The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method or composition of the invention, and vice versa. Furthermore, compositions of the invention can be used to achieve methods of the invention.

Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

The use of the term “or” in the claims is used to mean “and/or ” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.”

As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention overcomes deficiencies in the prior art by providing methods to determine if a patient has or is at risk of developing a renal disorder comprising evaluating the presence, absence or amount of one or more protein biomarkers (*e.g.*, a podocyte and/or tubular cell biomarker) produced by or present in the patient. In certain embodiments, if a biomarker is present in a sample taken from the patient (*e.g.*, a urine sample or a blood or plasma sample), then this indicates that the patient has or is at risk of developing a renal disorder. The present invention discloses the surprising discovery by the inventor that specific proteins, which are normally absent or present at very low concentrations in the urine of a healthy patient, can be found in higher concentrations in the urine and potentially in the blood, or one of its components such as serum or a RBC fraction, of patients who have specific renal disorders or are at risk of developing such disorders.

I. BIOMARKERS

The following biomarkers have been identified in the present invention as being indicative of a renal disorder. In certain embodiments the expression of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 16, 17, 18, 19, 20 or more of the biomarkers listed in Table 1 may be used to diagnose a renal disorder. In certain embodiments a fragment of a biomarker may also be used to diagnose or evaluate the progression of a renal disorder. For example, use of a fragment of a biomarker from listed in Table 1 may be used to diagnose a renal disorder.

In certain embodiments, the biomarker is selected from the group consisting of pigment epithelium-derived growth factor (PEDF), attractin, FGF 11, nestin, Dermcidin, fibulin 3, myosin Va, neuroligin-2, brain enriched hyaluronan binding protein, ryanodine receptor 2, ankyrin 3, FERM domain containing protein 4A, serum paraoxonase/aryltransferase, serum paraoxonase/aryltransferase 1 (PON1), neuropilin-2 (NP-2), and alpha2-macroglobulin. For example, in certain embodiments, the following primers may be used, *e.g.*, in a PCR assay to detect human PON1: human PON1 sense 5'-ATG GGA CTG GCA CTC TTC AG-3' (SEQ ID NO:1), human PON-1 antisense 5'-TGA AAG CCA GTC CAT TAG GC-3' (SEQ ID NO:2).

The expression of the biomarker may only be evaluated once in a patient, but preferably the expression of a biomarker evaluated in a subject more than once. In certain embodiments, the presence or amount of a biomarker is analyzed in a patient 2, 3, 4, 5, 6, 7, 8, 9, 10 or more times. The time between the analyzing may be weeks (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8 weeks), months (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 months) or years (*e.g.*, 1 or more years). The time between the analyzing will be decided by the patient's doctor and may vary depending on the individual patient and renal disease.

Table 1. Biomarkers for Renal Disorders

SP number	Protein name
P84336	Actin, cytoplasmic 1
P03996	actin. Aortic smooth muscle
O95622	adenylate cyclase type V
P04217	alpha 1B glycoprotein precursor
P02763	Alpha-1-acid glycoprotein 1
P01011	alpha-1-antichymotrypsin
P01009	Alpha-1-antitrypsin
P02765	Alpha-2-HS glycoprotein
P01023	Alpha-2-macroglobulin
P02760	AMBP protein precursor
P15144	Aminopeptidase N

SP number	Protein name
P22966	Angiotensin-converting enzyme, testis-specific isoform precursor (ACE-T) (Dipeptidyl carboxypeptidase I) (Kininase II) [Contains: Angiotensin-converting enzyme, testis-specific isoform, soluble form]
Q12955	Ankyrin 3
Q65ZQ1	Anti-colorectal carcinoma heavy chain
P46013	antigen KI-67
P02647	Apolipoprotein A1 prec.
P06727	apolipoprotein A-IV precursor
P05090	Apolipoprotein D
P02649	apolipoprotein E
P06396	ATP_Synthase beta chain
O75882	Attractin
Q96QZ7	BAI 1 associated protein 1
P98160	Basement membrane specific hep...
P61771	beta 2 microglobulin precursor
Q96GW7	brain enriched hyaluronan binding prot
O94833	bullous pemphigoid antigen 1
Q9ULB4	cadherin-9 precursor
P43155	carnitine o-acetyl transferase
P00450	ceruloplasmin
P10909	Clusterin precursor (Complement-associated protein SP-40,40) (Complement cytotoxicity inhibitor) (CLI) (NA1/NA2) (Apolipoprotein J) (Apo-J) (Testosterone-repressed prostate message 2) (TRPM-2)
P53618	Coatamer beta subunit
P12109	Collagen alpha-1(VI) chain precursor
P01024	Complement C3
P01028	Complement C4
P08603	Complement factor H
P25311	Cubulin precursor
P01034	cystatin C
P24855	Deoxyribonuclease I
P81605	Dermcidin
Q12805	EGF-containing fibulin-like matrix protein 1 (fibulin 3)
O60494	Endonuclease domain-containing 1 protein precursor
Q9HCU0	Endosialin precursor
Q9UNN8	Endothelial protein C receptor
P23588	eukaryotic translation initiation factor
P08294	Extracellular superoxide dismutase
Q9P2Q2	FERM domain containing protein 4A
Q92914	FGF 11 (fibroblast growth factor)
P01871	Fibrinogen beta chain precursor [Contains: Fibrinopeptide B]
P02679	Fibrinogen gamma chain
P23142	fibulin 1
P05062	Fructose-biphosphate aldolase B
P04004	Gelsolin precursor (Actin-depolymerizing factor) (ADF) (Brevin) (AGEL)
P14136	glial fibrillary acidic protein, astrocyte
Q16769	Glutaminy-peptide cyclotransferase

SP number	Protein name
Q07075	Glutamyl aminopeptidase (EAP) (Aminopeptidase A) (APA) (Differentiation antigen gp160) (CD249 antigen)
P22352	glutathione peroxidase 3 precursor
P04406	glyceraldehyde 3 phosphate dehydrogenase
P11217	glycogen phosphorylase muscle form
	golgi autoantigen
P00738	haptoglobin precursor
O94919	Haptoglobin-related protein precursor
Q96RW7	Hemicentin-1 precursor (Fibulin-6) (FIBL-6)
P69907	hemoglobin alpha subunit
P02042	hemoglobin delta
P68871	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin)
Q86YW2	HGFL(S) protein
P04196	histidine rich glycoprotein
Q13547	histone deacetylase 1
P17509	homeobox protein Hox-B6
Q86YZ3	Hornerin
Q6PIL8	Hypothetical protein (Immunoglobulin)
P01842	Ig gamma-1 chain C region
P01857	Ig gamma-2 chain C region
P01598	Ig heavy chain V-VIII region TIL
P00739	Ig kappa chain V-I region DEE
P01597	Ig kappa chain V-I region EU
P01765	Ig lambda chain C regions
P01859	Ig mu chain C region
Q6PJ95	IGHG1 protein
Q502W4	IGKC protein
Q6GMW4	IGLC2 protein
Q16270	Insulin-like growth factor binding ...
P19823	Inter-alpha-trypsin inhibitor heavy ...
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4 precursor (ITI heavy chain H4) (Inter-alpha-inhibitor heavy chain 4) (Inter-alpha-trypsin inhibitor family heavy chain-related protein) (IHRP) (Plasma kallikrein sensitive glycoprotein 120) (PK-120) (GP120)
P06870	Kallikrein
Q02241	kinesin like protein KIF23
P01042	Kininogen
Q13751	laminin beta 3 chain precursor
Q12802	LBC oncogene
P02750	Leucin rich alpha 2 glycoprotein
P07195	L-lactate dehydrogenase B chain
P98164	Low-density lipoprotein receptor-related protein 2 precursor (Megalin) (Glycoprotein 330) (gp330)
P10619	Lysosomal protective protein precursor (Cathepsin A) (Carboxypeptidase C) (Protective protein for beta-galactosidase)
P40925	Malate deshydrogenase
Q9NU22	Midasin
P80192	mitogen activated prot kinase

SP number	Protein name
P08571	monocyte differentiation CD14
Q9Y623	myosin heavy chain skeletal muscle fetal
Q9Y411	myosin Va (non muscle)
P48681	nestin
Q8NFZ4	neuroligin-2
O60462	neuropilin-2
P14543	Nidogen
P10153	Nonsecretory ribonuclease
Q14494	Nuclear factor erythroid 2 related factor 1
P49790	nuclear pore complex protein Nup153
Q15596	nuclear receptor coactivator 2
P12270	Nucleoprotein TPR
P55055	oxysterol receptor LXR-beta
O95153	Peripheral-type benzodiazepine receptor-associated protein 1
O00750	phosphatidylinositol-4 phosphate 3 kinase C2 domain containing betapolypeptide
P07205	Phosphoglycerate kinase
P36955	Pigment epithelium-derived factor
P02753	plasma retinol binding protein
P05154	Plasma serine protease inhibitor
P00747	plasminogen
Q13206	probable ATP dependent RNA helicase DDX10
O94919	Probable endonuclease
P01133	Pro-epidermal growth factor
P41222	Prostaglandin-H2 D-isomerase
Q16651	Prostatin
Q15084	Protein disulfide isomerase A6
P00734	Prothrombin
Q14644	Ras GTPase-activating protein 3
Q06495	renal sodium dependent phosphatase transport protein 2
Q92736	Ryanodine receptor 2
P04279	Semenogelin I
Q13523	serine/threonine-protein kinase PRP4
P02787	serotransferrin precursor
P02768	Serum albumin precursor
P35542	serum amyloid A-4 prot prec.
P02675	Serum amyloid P-component precursor (SAP) (9.5S alpha-1-glycoprotein)
P27169	serum paraoxonase / arylesterase
Q99835	smoothened homolog precursor
Q8NZM8	splicing fator Arg/Ser 16
O95425	Supervillin
Q9UMS6	synaptopodin-2 (myopodin)
Q9HBL0	tensin
Q8WY91	THAP domain prot 4
O75448	thyroid hormone receptor-associated protein complex 100 kda component
Q15643	thyroid receptor interacting protein 11
P02766	transthyretin (prealbumine)

SP number	Protein name
P34925	Tyrosine prot kinase RYK
P02248	Ubiquitin
Q05086	ubiquitin protein ligase E3A
P68543	UBX domain-containing protein 4
P07911	Uromodulin
P46939	utrophin
Q12907	Vesicular integral-membrane VIP36
P02743	Vitronectin precursor
Q8TD17	Zinc finger prot
O95218	Zinc finger prot 265
Q9BYN7	zinc finger protein 341
P25311	Zinc-alpha 2 glycoprotein precursor

II. RENAL DISORDERS

The present invention provides methods to determine if a patient has or is at risk of developing a renal disorder. Renal disorders that may be diagnosed with the present invention include, but are not limited to, a glomerular disease, a tubulo-interstitial disease, a vascular disease and transplanted kidney rejection. In certain preferred embodiments, acute renal failure may be predicted and/or diagnosed early using biomarkers of the present invention.

Glomerular diseases which may be diagnosed via the present invention include a minimal change disease, a focal or segmental glomerulosclerosis, a collapsing glomerulopathy, a membranous nephropathy, a membranoproliferative glomerulonephritis, a dense deposit disease, a cryoglobulinemia-associated glomerulonephritis, an IgA nephropathy, an Henoch-Schönlein disease, a postinfectious glomerulonephritis, a bacterial endocarditis, a pauci-immune crescentic glomerulonephritis, a Wegener granulomatosis, a microscopic polyangitis, a Churg-Strauss syndrome, an anti-GBM-antibody mediated glomerulonephritis, a lupus nephritis and a chronic allograft glomerulopathy. The glomerular disease may also have organized deposits which result from a amyloidosis, a monoclonal immunoglobulin deposition disease, a fibrillary glomerulonephritis and an immunotactoid glomerulopathy.

Tubulo-interstitial diseases which may be diagnosed via the present invention include an ischemic tubular injury, a medication-induced tubulo-interstitial nephritis, a toxic tubulo-interstitial nephritis, an infectious tubulo-interstitial nephritis, a bacterial pyelonephritis, a viral infectious tubulo-interstitial nephritis which results from a polyomavirus infection or an HIV infection, a metabolic-induced tubulo-interstitial disease, a mixed connective disease, a

cast nephropathy, a crystal nephropathy which may results from urate or oxalate or drug-induced crystals deposition, an acute cellular tubulo-interstitial allograft rejection, a tumoral infiltrative disease which result form a lymphoma or a post-transplant lymphoproliferative disease and an obstructive disease of the kidney.

5 Vascular disease which may be diagnosed via the present invention include a thrombotic microangiopathy, a nephroangiosclerosis, an atheroembolic disease, a mixed connective tissue disease, a polyarteritis nodosa, a calcineurin-inhibitor induced-vascular disease, an acute cellular vascular allograft rejection and an acute humoral allograft rejection.

10 Renal disorders which result from a hereditary or metabolic disease may also be diagnosed via the present invention. For example, the hereditary disease may be Alport's syndrome, thin membrane disease, Fabry's disease, a polycystic kidney disease, a renal disorder which results from a metabolic disease may be a diabetic nephropathy.

A. Podocytes and Renal disorders

15 Podocyte dysfunction is thought to be the starting point of segmental glomerulosclerosis (Gharahdaghi *et al.*, 1999). However, basic cell biological functions of the podocyte are not well known. The major reason of this incomplete knowledge is the difficulty to access and to study these cells. The morphological expression of the biological dysfunction of podocytes is quite limited and uniform. Indeed, podocytic ultrastructural lesions are stereotypical. They include foot process effacement, hypertrophy with
20 hyperplasia of organelles, reabsorption droplets, vacuolization of the cytoplasm, and, in most severe cases, podocyte drop out leading to the denudation of the glomerular basement membrane.

25 Small amounts of proteins are found in the urine of normal individuals (100 mg/24h or 75 mg/l). These urinary proteins derive from glomerular filtration, tubular excretion, shedding of microvesicles by tubular cells and vesiculation of podocytes.

30 Albumin and other plasma proteins account for about 90% of this physiological proteinuria. The remainder is principally Tamm-Horsfall protein, secretory IgA and a small amount of small molecular weight proteins. In addition, urinary excretion of small amounts of enzymes located in the cells of the renal tubules, and in particular in the brush border of the proximal tubules, is found in normal individuals.

Normal podocytes, or glomerular visceral epithelial cells, which are in direct contact with urinary space in the glomerulus, are known to release into urine small vesicles of various sizes lined by double membranes. It is not clear if these podocytes vesicles are shed from apical membrane of podocytes as ectosomes (or microparticles) or as exosomes. Ectocytosis (direct emission of membrane vesicles), is ubiquitous and has been described as enhanced in apoptotic or tumor cells upon cell activation. The composition of the ectosomes membrane proteins and lipids appears to be different from those of the original plasma membrane, indicating involvement of a selective sorting process during ectosome formation. Exosomes (unlike ectosomes) are membrane vesicles generated by a process involving endocytosis, endosome sorting into perinuclear multivesicular bodies (MVB) and exocytosis of MVBs. Exosome formation into the urinary space has been described for numerous epithelial cells like renal tubule epithelial cells. Podocyte vesicles have been shown to express the transmembrane protein Complement Receptor 1 (CR1 or CD35). The release of vesicle-bound CR1 into urine was demonstrated to be specific for podocytes and constant over time. These results were confirmed by Hara and colleagues, who identified these urinary vesicle-like particles by podocalyxin immunohistochemistry, a glycoprotein expressed on the apical cell membrane of podocytes (Pascual, 1994). The biological function of these vesicles in physiological conditions is not clear.

This vesiculation process may be increased in pathological conditions. Podocyte ultrastructural lesions, especially increased microvilli formation, are a constant feature of glomerulopathies characterized by increased proteinuria.

Without wishing to be bound by any theory, the data presented herein supports the hypothesis that these microvilli, localized in the urinary space as observed by electron microscopy analysis, may be excreted into urine as vesicles and represent a relevant part of the pathological proteinuria. Moreover, a modification of the protein pattern of these vesicles could reflect the physiopathological processes occurring in the damaged glomerulus. Since podocytes vesicles represent only a very small fraction of the physiological and pathological proteinuria, methods to enrich urinary samples in podocyte vesicles may be applied.

III. METHODS FOR DETECTION OF A BIOMARKER

In certain embodiments it may be preferable to test for the presence, absence or amount of a biomarker protein in a patient. However, in other embodiments, it may be desirable to test for the presence, absence or amount of a biomarker nucleotide (*e.g.*, a

biomarker mRNA) in a tissue sample from a patient to determine if the patient has or is at risk for a renal disorder. Analysis of a biomarker nucleotide may be performed using a tissue sample (*e.g.*, a blood, plasma or urine sample) from a patient using methods known to those of skill in the art (*e.g.*, gene chip analysis, reverse-transcription PCR or RT-PCR, Northern blot, *etc.*).

Detecting a biomarker protein may be performed via many different techniques, as known in the art. Immunodetection methods may be used to measure increased or decreased expression of a biomarker (*e.g.*, a podocyte biomarker) of the present invention in a biological sample from a patient. Examples of particular immunodetection methods include enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunoradiometric assay, immunohistochemistry, fluoroimmunoassay, chemiluminescent assay, electro-chemiluminescent assay, bioluminescent assay, and Western blot to mention a few. The immunoassay readout could be a mass spectrometer. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Doolittle and Ben-Zeev, 1999; Gulbis and Galand, 1993; De Jager *et al.*, 1993; Nakamura *et al.*, 1987, each incorporated herein by reference.

It is anticipated that proteins produced by podocytes may be detected in a biological sample from a patient using the methods described herein. For example, isolation of the podocytic vesicles of the whole urine may be performed using paramagnetic beads coated with a monoclonal antibody against CR1. Alternatively, analysis of the protein pattern of these podocytic vesicles (CR1-positive vesicles) may be done via polyacrylamide gel electrophoresis. Patterns of the tubular vesicles (CR1-negative vesicles) and of the total proteins in urine (soluble proteins) may be compared. Specific proteins may also be identified using mass spectrometers (*e.g.*, MALDI-TOF-TOF). Tubular vesicles (CR1-negative vesicles) may be isolated during the same procedure and separated from the urinary soluble proteins using ultracentrifugation. The enrichment in podocyte vesicles may be assessed by comparison with native urine (*e.g.*, with or without concentration and/or immunoabsorption on magnetic beads, with Western-blot analyses using podocalyxin, a transmembrane protein specific of the apical surface of the podocyte, and/or with a semiquantitative evaluation of the detected bands by densitometry analysis). The specificity of each sample (podocytic CR1-positive vesicles, tubular CR1-negative vesicles and CR1-

negative soluble proteins) may also be assessed with measurement of the CR1 levels using a specific ELISA.

In general, immunobinding methods involve measurement of the formation of immunocomplexes. Other methods include methods for isolating and purifying the biomarker protein from an organelle, cell, tissue or organism's samples. In these instances, the antibody removes or binds to the antigenic biomarker protein or message from a sample. The antibody will preferably be linked to a solid support, such as in the form of a column matrix, and the sample suspected of containing the message, protein, polypeptide and/or peptide antigenic component will be applied to the immobilized antibody. The unwanted components will be washed from the column, leaving the antigen immunocomplexed to the immobilized antibody to be eluted.

The immunobinding methods also include methods for detecting and quantifying the amount of an antigen component in a sample and the detection and quantification of any immune complexes formed during the binding process. Here, one would obtain a sample suspected of containing an antigen, and contact the sample with an antibody against the biomarker protein, and then detect and quantify the amount of immune complexes formed under the specific conditions.

In terms of antigen detection, the biological sample analyzed may be any sample that is suspected of containing an antigen, such as, for example, a tissue section or specimen, a homogenized tissue extract, a cell, an organelle, separated and/or purified forms of any of the above antigen-containing compositions, or even any biological fluid that comes into contact with the cell or tissue, including blood, serum, plasma and/or a tissue sample.

Contacting the chosen biological sample with the antibody under effective conditions and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the antibody composition to the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, *i.e.*, to bind to, any biomarker antigens present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any of those radioactive, fluorescent, biological and enzymatic tags. U.S. Patents concerning the use of such labels include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody and/or a biotin/avidin ligand binding arrangement, as is known in the art.

Any biomarker antibody employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined. Alternatively, the first antibody that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under effective conditions and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the antibody is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under effective conditions and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if this is desired.

One method of immunodetection uses two different antibodies. A first step biotinylated, monoclonal or polyclonal antibody is used to detect the target antigen(s), and a second step antibody is then used to detect the biotin attached to the complexed biotin. In

that method the sample to be tested is first incubated in a solution containing the first step antibody. If the target antigen is present, some of the antibody binds to the antigen to form a biotinylated antibody/antigen complex. The antibody/antigen complex is then amplified by incubation in successive solutions of streptavidin (or avidin), biotinylated DNA, and/or complementary biotinylated DNA, with each step adding additional biotin sites to the antibody/antigen complex. The amplification steps are repeated until a suitable level of amplification is achieved, at which point the sample is incubated in a solution containing the second step antibody against biotin. This second step antibody is labeled, as for example with an enzyme that can be used to detect the presence of the antibody/antigen complex by histoenzymology using a chromogen substrate. With suitable amplification, a conjugate can be produced which is macroscopically visible.

Another known method of immunodetection takes advantage of the immuno-PCR (Polymerase Chain Reaction) methodology. The PCR method is similar to the Cantor method up to the incubation with biotinylated DNA, however, instead of using multiple rounds of streptavidin and biotinylated DNA incubation, the DNA/biotin/streptavidin/antibody complex is washed out with a low pH or high salt buffer that releases the antibody. The resulting wash solution is then used to carry out a PCR reaction with suitable primers with appropriate controls. At least in theory, the enormous amplification capability and specificity of PCR can be utilized to detect a single antigen molecule.

It is recognized that other methods may be used to detect a biomarker of the present invention. For example, 2D gel electrophoresis (as described in, *e.g.*, Thongboonkerd *et al.* (2006); Stubberfield and Page (1999), cICAT (cleavable isotope-coded affinity tags) and/or iTRAQ (isobaric tags for relative and absolute quantification) (*e.g.*, Wu *et al.* (2006)) may be used with the present invention.

A. Western Blot Analysis

Western blot analysis is an established technique that is commonly employed for analyzing and identifying proteins. The proteins are first separated by electrophoresis in polyacrylamide gel, then transferred (“blotted”) onto a nitrocellulose membrane or treated paper, where they bind in the same pattern as they formed in the gel. The antigen is overlaid first with antibody, then with anti-immunoglobulin or protein A labeled with a radioisotope,

fluorescent dye, or enzyme. One of ordinary skill in the art would be familiar with this commonly used technique for quantifying protein in a sample.

B. ELISAs

As detailed above, immunoassays, in their most simple and/or direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and/or radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and western blotting, dot blotting, FACS analyses, and/or the like may also be used. One of ordinary skill in the art would be familiar with use of ELISAs and other immunohistochemical assays.

C. Immunohistochemistry

A biomarker antibody of the present invention may also be used in conjunction with both fresh-frozen and/or formalin-fixed, paraffin-embedded tissue blocks. The following methods for performing immunohistochemistry are provided as non-limiting descriptions of how the technique may be performed.

Briefly, 5- μ m histological sections are obtained from fresh-frozen biopsies. The sections are incubated with the primary antibody. Unbound antibody is removed by washing, and the bound antibody is detected either directly (if the primary antibody is labeled) or, indirectly using a fluorochrome-labeled secondary reagent. The staining is evaluated using fluorescence microscopy.

Using paraffin-embedded tissue blocks, 3- μ m histological sections are obtained from formaldehyde fixed paraffin-embedded biopsies. These are deparaffinated and then endogenous peroxidase is blocked with 0.3% H_2O_2 in methanol for 30 min. The sections are digested with 0.05% proteinase K in PBS for 2 min, and incubated with the primary antibody. Then they are incubated with the labeled, secondary binding ligand, or antibody. For detection, a substrate-chromogen system is used. Counterstaining is by hematoxylin Mayer, and dehydration by using decreasing alcohol concentrations. Stained sections are mounted with permanent mounting medium and the tissues are examined with a microscope.

Another procedure for study by immunohistochemistry (IHC) may use blocks such as blocks prepared from a tumor biopsy. The method of preparing tissue blocks from these

particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and/or is well known to those of skill in the art (Brown *et al.*, 1990; Abbondanzo *et al.*, 1999; Allred *et al.*, 1990).

Briefly, frozen-sections may be prepared by rehydrating 50 ng of frozen "pulverized" tissue at room temperature in phosphate buffered saline (PBS) in small plastic capsules; pelleting the particles by centrifugation; resuspending them in a viscous embedding medium (OCT); inverting the capsule and/or pelleting again by centrifugation; snap-freezing in -70°C isopentane; cutting the plastic capsule and/or removing the frozen cylinder of tissue; securing the tissue cylinder on a cryostat microtome chuck; and/or cutting 25-50 serial sections.

Permanent-sections may be prepared by a similar method involving rehydration of the 50 mg sample in a plastic microfuge tube; pelleting; resuspending in 10% formalin for 4 hours fixation; washing/pelleting; resuspending in warm 2.5% agar; pelleting; cooling in ice water to harden the agar; removing the tissue/agar block from the tube; infiltrating and/or embedding the block in paraffin; and/or cutting up to 50 serial permanent sections.

D. Protein Array Technology

In certain embodiments, a biomarker of the present invention may be analyzed using a protein array and/or a protein biochip. Protein array technology allows high-throughput screening for gene expression and molecular interactions. Protein arrays appear as new and versatile tools in functional genomics, enabling the translation of gene expression patterns of normal and diseased tissues into protein product catalog. Protein function, such as enzyme activity, antibody specificity, and other ligand-receptor interactions and binding of nucleic acids or small molecules can be analyzed on a whole-genome level.

1. Protein Biochip Assays

These arrays, which contain tens, hundreds or thousands of different proteins or antibodies spotted onto glass slides or immobilized in tiny wells, allow one to examine the biochemical activities and binding profiles of a large number of proteins at once. To examine protein interactions with such an array, a labeled protein is incubated with each of the target proteins immobilized on the slide, and then one determines which of the many proteins the labeled molecule binds.

The basic construction of protein chips has some similarities to DNA chips, such as the use of a glass or plastic surface dotted with an array of molecules. These molecules can be DNA or antibodies that are designed to capture proteins. Defined quantities of proteins are immobilized on each spot, while retaining some activity of the protein. With fluorescent markers or other methods of detection revealing the spots that have captured these proteins, protein microarrays are being used as powerful tools in high-throughput proteomics and drug discovery.

Glass slides are still widely used, since they are inexpensive and compatible with standard microarrayer and detection equipment. However, their limitations include multiple-based reactions, high evaporation rates, and possible cross-contamination.

Matrix slides offer a number of advantages, such as reduced evaporation and no possibility of cross-contamination, but they are expensive. Nanochips for proteomics have the same advantages, in addition to reduced cost and the capability of multiple-component reactions.

A well-known protein chip is the ProteinChip by Ciphergen Biosystems Inc. (Fremont, CA). The ProteinChip is based on the surface-enhanced laser desorption and ionization (SELDI) process. Known proteins are analyzed using functional assays that are on the chip. For example, chip surfaces can contain enzymes, receptor proteins, or antibodies that enable researchers to conduct protein-protein interaction studies, ligand binding studies, or immunoassays. With state-of-the-art ion optic and laser optic technologies, the ProteinChip system detects proteins ranging from small peptides of less than 1000 Da up to proteins of 300 kDa and calculates the mass based on time-of-flight (TOF).

The ProteinChip biomarker system is a protein biochip-based system that enables biomarker pattern recognition analysis to be done. This system allows researchers to address important clinical questions by investigating the proteome from a range of crude clinical samples (*i.e.*, laser capture microdissected cells, biopsies, tissue, urine, and serum). The system also utilizes biomarker pattern software that automates pattern recognition-based statistical analysis methods to correlate protein expression patterns from clinical samples with disease phenotypes.

Some systems can perform biomarker discovery in days and validation of large sample sets within weeks. A robotics system accessory automates sample processing,

allowing hundreds of samples to be run per week and enabling a sufficient number of samples to be run, which provides high statistical confidence in comprehensive studies for marker discovery and validation.

2. Microfluidic Chip-Based Immunoassays

5 Microfluidics is a very important innovation in biochip technology. Since microfluidic chips can be combined with mass spectrometric analysis, a microfluidic device has been devised in which an electrospray interface to a mass spectrometer is integrated with a capillary electrophoresis channel, an injector, and a protein digestion bed on a monolithic substrate (Wang *et al.*, 2000). This chip thus provides a convenient platform for automated
10 sample processing in proteomics applications.

These chips can also analyze expression levels of serum proteins with detection limits comparable to commercial enzyme-linked immunosorbent assays, with the advantage that the required volume sample is markedly lower compared with conventional technologies.

Biosite (San Diego) manufactures the Triage protein chip that simultaneously
15 measures 100 different proteins by immunoassays. The Triage protein chip immunoassays are performed in a microfluidic plastic chip, and the results are achieved in 15 minutes with picomolar sensitivities. Microfluidic fluid flow is controlled in the protein chip by the surface architecture and surface hydrophobicity in the microcapillaries. The immunoassays utilize high-affinity antibodies and a near-infrared fluorescent label, which is read by a
20 fluorometer.

3. Tissue Microarray Technology

Tissue microarray technology provides a high-throughput approach for linking genes and gene products with normal and disease tissues at the cellular level in a parallel fashion. Compared with classical *in situ* technologies in molecular pathology that are very time-
25 consuming, tissue microarrays provide increased throughput in two ways: up to 1000 tissue specimens can be analyzed in a single experiment, either at the DNA, RNA, or protein level; and tens of thousands of replicate tissue microarrays can be generated from a set of tissues. This process provides a template for analyzing many more biomarkers than has ever been possible previously in a clinical setting, even using archival, formalin-fixed specimens.

4. Nanoscale Protein Analysis

Most current protocols including protein purification and automated identification schemes yield low recoveries that limit the overall process in terms of sensitivity and speed. Such low protein yields and proteins that can only be isolated from limited source material (e.g., biopsies) can be subjected to nanoscale protein analysis: a nanocapture of specific proteins and complexes, and optimization of all subsequent sample-handling steps, leading to a mass analysis of peptide fragments. This focused approach, also termed targeted proteomics, involves examining subsets of the proteome (e.g., those proteins that are specifically modified, bind to a particular DNA sequence, or exist as members of higher-order complexes or any combination thereof). This approach is used to identify genetic determinants of cancer that alter cellular physiology and respond to agonists.

Multiphoton detection, by Biotrace Inc. (Cincinnati), can quantify subzeptomole amounts of proteins and can be used for diagnostic proteomics, particularly for cytokines and other low-abundance proteins. Biotrace is also developing supersensitive protein biochips to detect concentrations of proteins as low as 5 fg/ml (0.2 attomole/ml), thereby permitting sensitivity that is 1000 times greater than current protein biochips.

E. *In situ hybridization*

The active local synthesis of biomarker protein, in the kidney, may be confirmed by the detection of mRNA using *in situ* hybridization.

Briefly, a PCR-product representing the biomarker protein cDNA is cloned into an expression vector. The vector is linearized enzymatically, followed by phenol extraction and precipitation under RNase free conditions. Run-off digoxigenine (DIG) labelled sense- and anti-sense mRNA transcripts are transcribed using polymerase.

Paraffin-embedded sections are deparaffinated, washed and rehydrated through the graded series of ethanol. The sections are postfixed in 4% paraformaldehyde in TBS, washed in TBS and digested with proteinase K in TE buffer. After washing in TBS, the digestion is stopped by incubating the sections in TBS. The sections are prehybridized in 4 x SSC and 50% formamide. The probes are diluted in hybridization buffer and incubated before adding to the sections. The sections are then incubated. After incubation, any unbound probe is washed off. The sections are incubated with anti-DIG-AP Fab fragments. Then the sections are washed in TBS and colour detection is performed. The sections are background stained

with Mayer's hematoxylin. Negative controls include adjacent sections of normal renal tissue incubated with sense mRNA probe.

IV. KITS

The present invention also contemplates the generation of kits relating to the identification of a biomarker of the present invention. The kit preferably includes a container means and an antibody which selectively recognizes the biomarker. In certain embodiments, an immunoassay kit such as an ELISA kit, may be produced to test for the presence or absence of a biomarker or the present invention. Kits of the present invention may be used to determine if a patient has or is at risk of a renal disorder.

Kits to differentiate tubulo-interstitial diseases from glomerular diseases (*e.g.*, a tubulo-interstitial nephritis test), kits to differentiate the different types of glomerular diseases (*e.g.*, a minimal change test, a focal and segmental glomerulosclerosis test, a membranous glomerulopathy test, *etc.*), kits to identify vascular diseases (*e.g.*, a thrombotic microangiopathy test) and kits for allograft nephropathy (*e.g.*, an acute allograft rejection test) may be prepared via the present invention.

V. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Identification of Biomarkers for Renal Disorders

Urine Collection

Urines were obtained from 19 healthy individuals (7 males and 12 females, age 37.9 ± 8.7 years) and from 9 patients with a renal biopsy-proven renal disease (6 male, 3 females, age 45 ± 17.6 years, serum creatinine 203 ± 79 $\mu\text{mol/l}$). The corresponding renal diseases are

listed below in table 4. Urine samples obtained from patients were collected the day of the renal biopsy or in the 5 days following the renal biopsy.

Table 4.

Patient	Gender	Age(years)	Screat (μ mol/l)	Proteinuria	Microhaematuria	Renal pathology
1.	M	53	82	25g/d	no	Membranous nephropathy
2.	M	28	110	0.5/d	yes	IgA GN
3.	F	41	199	++++	no	Lupus nephritis IV(A)
4.	M	22	250	8g/d	no	FSGS I
5	F	24	320	1g/d	ND	Benign nephroangiosclerosis
6.	M	59	279	5.2g/d	yes	Malignant nephroangiosclerosis and FSGS II
7.	M	54	145	27g/d	yes	Diabetes and FSGS II
8	M	74	240	+	yes	Tubulo-interstitial nephritis with granuloma
9.	F	50	200	0.5g/d	no	Tubulo-interstitial nephritis

Patient Characteristics

First morning urines were collected into sterile 500-ml plastic tubes containing 0.05% sodium azide. Protease inhibitor cocktail (Complete mini protease inhibitor cocktail, Roche, Basel, Switzerland) and antibiotics (Pen-Strep, Gibco, Invitrogen, CA, USA) were added to
5 avoid proteolysis and bacterial growth in the sample.

Urine specimens were centrifuged at 2500 g for 15 minutes at room temperature immediately after collecting to remove whole cells and cell debris. The supernatants were frozen at -80°C, and stored until concentration and analysis.

Each patient gave informed consent prior to enrolment. The local institutional ethical
10 committee board approved the clinical protocol.

Urine Concentration

Each urine sample was spun at 3'500 g for 30 minutes at room temperature using a 10-kDA cutoff centrifugal column (Centricon plus-80, Amicon, Beverly, MA, USA) until approximately 1/100 of initial volume remained. The concentrated urine was desalted and
15 buffered with an equal volume of Tris-HCl 10mM pH 8.5 in the same column at 3'500 g for 30 minutes. The concentrated urines of the 19 healthy individuals were pooled into 7 samples of 2-3 individuals (control samples). The protein amounts in urine concentrates were measured using a Biorad protein microassay based on the method of Lowry (1951).

Enrichment of the Urinary Samples in Podocyte Vesicles

20 The concentrated urine samples were incubated with paramagnetic beads coated with a monoclonal antibody against Complement Receptor type 1 (3D9, purified from ascites according to the method described by Quadri et al (Quadri and Schifferli, 1992). In brief, 1ml magnetic beads (Dynabeads M-450 Tosylactivated, Bio-Rad Laboratories Hercules, CA) were prepared according to the manufacturer's instructions and incubated with 400 µg of
25 anti-CR1 mAb 3D9 in PBS pH 7.4 (1ml final volume) overnight at 37°C with gentle mixing. The tube was then placed in a magnetic bead separator and the supernatant was removed carefully using a pipette. The immunocoated magnetic beads were washed three times in three different wash buffers according to the manufacturer's instructions and immediately incubated with 1ml concentrated and ultracentrifuged urine sample for 4 hours at 37°C with

gentle mixing. The CR1 positive podocyte vesicles immunoabsorbed on the beads were then isolated with a magnetic bead separator and eluted in 1ml of lysis buffer (PBS pH 7.4 containing 5 mM EDTA pH 8.0, Triton 1%, SDS 0.1% and 1mM PMFS) overnight at 4°C with gentle mixing. The lysis buffer containing the CR1-positive podocyte lysate was then separated from the beads and centrifuged at 16'000 g for 20 minutes at 4°C. The supernatant containing the soluble proteins extracted from the podocyte vesicles was recovered and the protein concentration was measured using the Lowry method.

Podocyte vesicles enrichment was assessed using immunoblot analysis. Original urines, samples obtained after concentration and samples obtained after concentration and immunoabsorption on magnetic beads were compared. An equal proportion of the original urine volume was loaded onto gels. The proteins were separated by SDS-PAGE and transferred onto PVDF membranes as described by (Towbin *et al.*, 1979). After blocking with 5% milk (1h), membranes were probed overnight with podocalyxin (monoclonal anti-human podocalyxin antibody; 0.2 µg/ml, R and D systems, Minneapolis, MN, USA). Peroxydase-conjugated, affinity-purified donkey anti-rabbit (1:100'000) (Jackson Immuno-Research, West Grove, PA, USA) was used for 90 min at room temperature. The antibody-antigen reactions were visualized by using enhanced chemiluminescence plus Western Blotting Kit (Roche, Basel, Switzerland) and light-sensitive film (Kodak Biomax XAR, Rochester, NY, USA). The density of the bands was quantified by National Institutes of Health ImageJ software.

SDS-PAGE and In-Gel Digestion

Supernatant proteins (80-100 µg) obtained from CR1 immunoabsorption were separated by SDS-PAGE on homemade 7.5% and 15% T Tris-Glycine gels (8 x 5 x 0.15 cm). After the migration, gels were stained with an MS-compatible silver stain (Blum *et al.*, 1987) and all the well-stained bands were cut. The cut bands were then destained with 15mM K₃Fe(CN)₆, 50mM Na₂S₂O₃ and washed with Milli-Q water (Millipore, Billerica, MA) using standard protocols (Gharahdaghi, 1999). The gel pieces were dehydrated in 100% CH₃CN and dried in a vacuum centrifuge. The proteins were digested in-gel with trypsin using standard protocols (Scherl, 2002). Peptides were then extracted with 1% TFA followed by 50% CH₃CN, 0.1% TFA. The combined extracts were concentrated by vacuum centrifugation.

LC-MS/MS

Peptides extracted following in-gel digestion were analyzed by LC-MS/MS as previously described (Burgess *et al.*, 2006). Briefly, they were dissolved in 9 μ l 5% CH₃CN, 0.1% formic acid and 5 μ l was loaded for LC-MS/MS analysis. A precolumn (100 μ m inner diameter, 2-3.5 cm long) was connected directly to an analytical column (75 μ m inner diameter, 9-10 cm long). Both columns were packed in-house with 5 μ m, 3 Å Zorbax Extend C-18 (Agilent). A gradient from 4 to 56% solvent B (80% CH₃CN, 0.1% formic acid) in solvent A (5% CH₃CN, 0.1% formic acid) was developed over 15 min at a flow rate of approximately 300 nl/min. The eluate was sprayed directly into the nanoESI source of an LCQ DecaXP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) with a spray voltage of 1.8-2.2 kV. Data dependant acquisition was used to automatically select 2 precursors for MS/MS from each MS spectrum (m/z range 400-1600). MS/MS spectra were acquired with a normalized collision energy of 35%, an activation Q of 0.25 and an isolation width of 4 m/z . The activation time was 30 ms. Dynamic exclusion was applied with a repeat count of 2, an exclusion time of 30 s, and an exclusion peak width of ± 1.5 Da. Wideband activation was also applied. Maximum injection times of 50 ms and 200 ms were used for MS and MS/MS acquisitions, respectively, and the corresponding automatic gain control targets were set to 10⁸.

Data Extraction and Data Interrogation

Peak lists were generated using Bioworks 3.1 software (Thermo Finnigan, San Jose, CA). The resulting data files from each analysis were automatically combined into a single text file. The resulting peak lists were searched against the UniProt combined Swiss-Prot and TrEMBL database (Release 50.0) without species restriction using Mascot operating on a local server (version 2.1.04, Matrix Sciences, U.K.) and Phenyx Virtual Desktop (Gene Bio, Switzerland). Mascot was used with average mass selected, a precursor mass error of 2 Da and a peptide mass error of 1.0 Da. Trypsin was selected as the enzyme, with a single potential missed cleavage. ESI ion trap was selected as the instrument type and oxidized methionine as a variable modification. Only the peptides that were above the threshold in Mascot searches (< 5% probability of false match for each peptide above this score) were considered. For Phenyx, ion trap was selected for the instrument type and LCQ for the algorithm. Two search rounds were used both with trypsin selected as the enzyme and oxidized methionine as a variable modification. In the first round, one missed cleavage was

allowed and the normal cleavage mode was used. This round was selected in “turbo” search mode. In the second round, two missed cleavages were allowed and the cleavage mode was set to half-cleaved. The minimum peptide length allowed was 6 amino acids and the parent ion tolerance was 2.0 Da in both search rounds. The acceptance criteria were slightly lowered in the second round search (round 1: AC score 7.0, peptide Z-score 7.0, peptide *p* value 1E-6; round 2: AC score 7.0, peptide Z-score 6.0, peptide *p* value 1E-5). Proteins that were identified as human proteins with 3 or more peptides from both Mascot and Phenyx were accepted to be true matches. Matches with fewer than 3 peptides were manually validated.

Enrichment of podocyte vesicles from urinary samples

The enrichment in podocyte vesicles was assessed by comparison of native urine, samples obtained after concentration and samples obtained after concentration and immunoabsorption on magnetic beads. Western-blot analyses were performed using podocalyxin, a transmembrane protein specific of the apical surface of the podocyte, and a semiquantitative evaluation of the detected bands was done by densitometry analysis. Podocalyxin, which was not detectable in native urine sample, increased after concentration of normal urine samples and after concentration and immunoabsorption on magnetic beads. The increase after concentration and immunoabsorption was of about 13-20 fold in comparison with samples after concentration only.

In certain experiments, the peak lists were searched against the UniProt combined Swiss-Prot and TrEMBL database restricted to human entries using Phenyx Virtual Desktop (Gene Bio, Switzerland) as previously described. The acceptance criteria were more stringent than for the search of the Swiss Prot database alone (round 1: AC score 16.0, peptide Z-score 8.0, peptide *p*-value 1 E-7; round 2: AC score 10.0, peptide Z-score 7.0, peptide *p*-value 1 E-6).

1. PEDF

PEDF (pigment epithelium-derived factor, 46kDa) was first identified in conditioned medium of human fetal retinal pigment epithelium cells in culture in 1987 by Tombran-Tink and Johnson, is a multipotent factor (neurotrophic and neuroprotective factor, antiangiogenic factor, implicated in cell cycle and aging). The protein is detected in normal kidney (human, murine, rat) and is immunolocalized to the renal tubular epithelial cells of both cortex and

medulla. In the rat kidney it is also detected in glomeruli with a staining suggestive of podocyte localization. (Tombran-Tink and Barnstable 2003; Gettins *et al.* 2002).

The protein was detected in normal human concentrated urine by Western blot analysis. An upregulation of the urinary protein levels was demonstrated in tubulo-interstitial nephritis (6/9) by Western blot analysis and ELISA. No or only slight modulation of the urinary protein levels was observed in glomerulonephritis (25/31). and in vascular disease (5/6).

The inventors detected the protein in tubular cells (likely collecting tubes) by immunohistochemistry. No, or only a weak, signal was detected in glomerular podocytes. In tubulo-interstitial nephritis, a decrease of the tubular immunostaining was observed (2/2). In glomerulopathies, the tubular immunostaining was similar to the normal controls (2/2). The hypothesis is that PEDF, in physiological condition, is filtered through glomerular barrier and reabsorbed by tubular cells. In case of tubular lesions, PEDF is not more reabsorbed but excreted in high amount in urine.

2. FGF-11

FGF-11 (Fibroblast growth factor 11 or FGF3 Fibroblast growth factor homologous factor 3, 25kDa) is expressed in the developing and adult nervous systems, suggesting a role in nervous system development and function (Smallwood *et al.*, 1996).

The protein was detected in normal human concentrated urine by Western blot analysis. No renal synthesis was observed with PCR.

An upregulation of the urinary protein levels was observed in TIN (6/6) by Western blot analysis. A modulation of the urinary protein levels was observed in GN (IgA, LED, membranous GN and FSGS) by Western blot analysis.

3. Neuroligin 2

Neuroligin 2 (90kDa) is found to be expressed exclusively in the brain with no detectable specific signal in other tissues such as muscle (Scheiffele *et al.*, 2000).

The protein was detected in normal human concentrated urine by Western blot analysis. A renal synthesis was demonstrated with PCR analysis.

A modulation of the urinary protein levels in glomerulopathies (FSGS and membranous GN) was observed by Western blot analysis. It is interesting to note that this modulation was similar to that observed with FGF-11 (for the same cases and in the same technical conditions).

4. Fibulin 3

Fibulin 3 (54 kDa), a protein with an important role in microfibrillar structures, is detected in the kidney around medium-sized vessels and some tubules, but only in restricted regions of the cortex (Giltay *et al.*, 1999).

The protein was detected in normal human concentrated urine by Western blot analysis. A renal synthesis was demonstrated with PCR analysis. A modulation of the urinary protein levels in glomerulopathies (FSGS and membranous GN) was observed by Western blot analysis.

5. PON-1

Serum paraoxonase/aryltransferase 1 (PON1) has not been previously detected in human urine, even in the largest urinary proteome catalogs obtained using different methodologies. PON-1 was identified as a human protein with only one peptide from Mascot. However, the spectrum analysis of this peptide strongly correlated with human PON-1.

Immunoblot Analyses for PON-1

PON-1 was detected in only one urine sample. This urine was obtained from a patient with severe lupus nephritis and renal insufficiency. In order to confirm the presence of PON1 in this pathological urine and to assess the possible modulation of the protein levels in case of renal disease, Western-blot analysis was performed on pathological and normal urines.

Protein samples from concentrated urines (40 µg) were separated by SDS-PAGE. Urines obtained from 3 normal individuals and from 3 patients with biopsy-proven renal diseases were analyzed. Proteins were transferred to PVDF membranes as described by Towbin *et al.* (1979). After blocking with 5% milk (1h), membranes were probed overnight with polyclonal rabbit anti-human PON-1 diluted 1/1000 (Atlas Antibodies AB, Stockholm, Sweden). Peroxydase-conjugated, affinity-purified donkey anti-rabbit (1:100'000) (Jackson Immuno-Research, West Grove, PA, USA) was used for 90 min at room temperature. The

antibody-antigen reactions were visualized by using enhanced chemiluminescence plus Western Blotting Kit (Roche, Basel, Switzerland) and light-sensitive film (Kodak Biomax XAR, Rochester, NY, USA). Whole kidney tissue extract (40 µg) was also analysed. Liver (40 µg) was used as positive control.

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An immunoreactive band of approximately 45kDa, corresponding to the known PON-1 molecular weight, was detected in all the urine samples. The most intense signal was observed with the pathological urine from patient with severe lupus nephritis. These results confirm the presence of PON-1 protein in human urine and the likely modulation in case of renal disease.

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Normal whole kidney tissue extract was analyzed using the same method. Two different bands were detected, one of similar molecular weight than the one detected in urine, and a smaller one, which was of similar molecular weight than the ones detected in liver extract. These two bands may correlate with urine and blood which are both found in the renal tissue. It is also interesting to note that MW of urinary PON-1 is slightly higher than MW of plasma PON-1 since the inventors might have expected a lower MW due to urinary degradation. Additional components, such as sugars, could perhaps explain this slightly higher MW.

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Immunochemistry Examination of PON-1

Normal renal tissue was obtained from normal parts of four nephrectomies performed for renal carcinoma. Histological sections (3 µm) were obtained from formaldehyde-fixed paraffin-embedded tissues. Sections were deparaffinized and endogenous peroxidase was blocked with a peroxidase blocking solution (DAKO, Gostrup, Denmark) for 10 minutes at RT. The sections were treated for antigen retrieval with microwave irradiation in citrate buffer saline for 10 min, and incubated with the primary polyclonal rabbit anti-human PON-1 antibody (Atlas Antibodies AB, Stockholm, Sweden) at a 1:200 dilution overnight at 4°C. Then, they were incubated with a horseradish peroxidase -conjugated goat anti-rabbit antibody (HRP Envision+, Dako, Gostrup, Denmark) for 30 min at room temperature. For detection, the liquid diaminobenzidine substrate-chromogen system (Dako, Gostrup, Denmark) was used according to the manufacturer's instructions. Counterstaining was

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performed using hematoxylin Mayer, and dehydration by using decreasing alcohol concentrations. Stained sections were mounted with permanent mounting medium (Eukitt, Electron microscopy sciences, Hatfield, PA, USA) and the tissues were examined with a Zeiss microscope (Zeiss, Oberkochen, Germany).

- 5 Negative controls included adjacent sections of normal renal tissue incubated with rabbit IgG fraction, bovine serum albumin and buffer alone.

 PON-1 protein was detected exclusively at three different locations in the kidney: the glomeruli, the tubules and the vessels. In glomeruli, PON-1 was detected in podocytes. In addition to the podocytes, some staining was detected in parietal epithelial cells of the
10 Bowman's capsule as well as in the urinary Bowman's space, likely corresponding to the primitive urine as suggested by the positive staining observed in the tubular pole of the glomerulus. Glomerular endothelial and mesangial cells were negative. In tubules, PON-1 was detected in the cytoplasm of distal tubules, both in cortex and in medulla.. In addition to this cytoplasmic staining, a luminal positive staining was noted in some of the cortical and
15 medullary tubules, most likely corresponding to urine. In the vessels, PON-1 protein was detected in the arteries, in the smooth muscle cells of the media. Large and small arteries as well as arterioles of both cortex and medulla were positive. A positive but weaker staining was also detected in the endothelial cells of the cortical and medullary arteries. In addition, a positive staining was detected in the lumen of vessels, likely corresponding to the serum
20 components. No staining was detected in the endothelial cells of veins and peritubular capillaries. The other structures in the cortex and medulla including the interstitial cells were negative. No glomerular, tubular or vascular staining was detected with rabbit immunoglobulin (Ig) G used as a negative control.

RNA isolation and RT-PCR Analyses

- 25 According to the literature, PON-1 expression has never been described in the kidney (James *et al.*, 2006). In order to analyze the possible synthesis of PON-1 in the normal human kidney, RT-PCR analysis was performed using human-specific primers.

 Normal human renal tissue was obtained from normal parts of 3 nephrectomies performed for renal cell carcinoma. In addition, for one nephrectomy, cortex was separated
30 from medulla. Tissues were disrupted with a Tissue Taeror (Biospec Products, Racine, WI). Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) and quantified by

spectrophotometry. An equal amount (2µg) of total RNA was reverse transcribed with Superscript II reverse transcriptase (Life Technologies) in the presence of random hexamers according to the manufacturer's instructions. PCR was accomplished using a kit (1st Strand cDNA Synthesis Kit for RT-PCR (AMV), Roche Applied Science, Indianapolis, IN, USA) and the following primers: human PON1 sense 5'-ATG GGA CTG GCA CTC TTC AG-3' (SEQ ID NO:1), human PON-1 antisense 5'-TGA AAG CCA GTC CAT TAG GC-3' (SEQ ID NO:2).

Primer pairs were obtained from Clontech (La Jolla, CA, USA). Mock reactions devoid of cDNA served as negative controls. PCR products were analyzed by agarose gel electrophoresis followed by staining with ethidium bromide.

Experiments confirmed that PON-1 mRNA is expressed in the normal human kidney, both in the cortex and in the medulla.

6. Neuropilin-2

Neuropilin-2 (NP-2) is a cell surface transmembrane protein originally characterized as a receptor for the type 3 semaphorins, and more recently for a number of vascular endothelial growth factor (VEGF) isoforms (Cohen *et al.*, 2002).

In the developing mouse embryo, NP-2 expression was detected in the CNS, lung, mesenteric, muscular, and submucosal plexuses of the intestine and in bone and cartilage (Chen *et al.*, 1997; Giger *et al.*, 1998). The expression pattern of NP-2 in adult mice or in human tissue outside the CNS is not well documented. NP-2 was reported to be expressed by a subset of serotonin-expressing neuroendocrine cells in the stomach and small and large intestines and in pancreas in the islet periphery in co-localization with glucagons-expressing (alpha) cells (Cohen *et al.*, 2001; Cohen *et al.*, 2002).

This protein was detected by immunohistochemistry in glomerular podocytes, in endothelial cells of renal vessels and in collecting ducts of the medulla. The protein was not detected in normal human concentrated urine by Western blot analysis. No renal synthesis was observed with PCR.

7. Alpha2-macroglobulin

α_2 macroglobulin (α_2 M) is a 720 kDA glycoprotein which is mainly synthesized in the liver (de Sain-van der Velden, 1998). Increased levels of α_2 M are well described in patients with nephrotic syndrome regardless of the primary disease (Vazirdi, 1994). α_2 M, which was identified in human urine, has been suggested to be a marker of glomerular diseases. Thus, Tencer et al, which investigated 199 patients with renal biopsy proven-glomerulopathies suggested that the α_2 M selectivity index (SI) could be used to effectively distinguish two types of glomerular disorders (minimal change nephropathy and crescentic necrotizing glomerulonephritis) from all the other analyzed glomerular diseases Tencer, 1998). In another study, Ito et al analyzed urine samples obtained from diabetic patients with retinopathy using a sensitive radioimmunometric assay to measure urinary α_2 M levels (Ito, 1995). They found that the highest urinary α_2 M excretion rates were observed in Albustix-positive patients followed by Albustix-negative patients and the healthy controls.

The protein was detected in normal human concentrated urine by Western blot analysis. A modulation of the urinary protein levels in glomerulopathies was observed by Western blot analysis.

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All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS

1. A method of assessing whether an individual has or is at risk for developing a renal disorder comprising the steps of:
 - a) obtaining a biological sample from the individual;
 - 5 b) analyzing the sample to determine the presence, absence or amount of one or more biomarkers selected from the group consisting of the biomarkers of Table 1; and
 - c) assessing from said presence, absence or amount of the one or more biomarkers whether the individual has or is at risk for developing a renal

10 disorder.
2. The method of claim 1, wherein the analyzing comprises determining the presence or amount of one or more of pigment epithelium-derived factor, Attractin, FGF 11 (fibroblast growth factor), nestin, Dermcidin, EGF-containing fibulin-like matrix

15 protein 1 (fibulin 3), myosin Va (non muscle), neuroligin-2, brain enriched hyaluronan binding protein, Ryanodine receptor 2, Ankyrin 3, serum paraoxonase/aryltransferase 1 (PON1), neuropilin-2 (NP-2), alpha2-macroglobulin or FERM domain containing protein 4A.
3. The method of claim 1, wherein the analyzing comprises determining the presence or

20 amount of one or more of pigment epithelium-derived factor, FGF 11 (fibroblast growth factor), EGF-containing fibulin-like matrix protein 1 (fibulin 3), neuroligin-2, serum paraoxonase/aryltransferase 1 (PON1), neuropilin-2 (NP-2), or alpha2-macroglobulin.
4. The method of claim 2, wherein the analyzing comprises determining the presence or

25 amount of two or more of the biomarkers.
5. The method of claim 2, wherein the analyzing comprises determining the presence or amount of three or more of the biomarkers.
6. The method of claim 2, wherein the analyzing comprises determining the presence or amount of four or more of the biomarkers.

7. The method of claim 2, wherein the analyzing comprises determining the presence or amount of five or more of the biomarkers.
8. The method of claim 2, wherein the analyzing comprises determining the presence or amount of six or more of the biomarkers.
- 5 9. The method of claim 2, wherein the analyzing comprises determining the presence or amount of seven or more of the biomarkers.
10. The method of claim 1, wherein the sample is a blood sample, a urine sample, a kidney biopsy sample, a serum sample or a plasma sample.
11. The method of claim 10, wherein the sample is a blood sample, a serum sample or a
10 plasma sample.
12. The method of claim 10, wherein analyzing the sample comprises performing one or more of an ELISA, an immunoassay, RT-PCR, a radioimmunoassay (RIA), an immunoradiometric assay, a fluoroimmunoassay, an immunoassay, a chemiluminescent assay, a bioluminescent assay, a gel electrophoresis, a Western blot
15 analysis, a silver-stained gel analysis, an *in situ* hybridization assay, an immunohistochemistry assay, a protein biochip assay, a microfluidic chip-based immunoassay, a mass spectrometry assay, a GC/MS assay, a LC-MS/MS assay, a MALDI tof mass spectroscopy assay, SELDI tof mass spectroscopy assay, or a HPLC assay thereon.
- 20 13. The method of claim 12, wherein the analyzing the sample comprises performing an immunoassay thereon.
14. The method of claim 1, wherein the renal disorder is a glomerular disease.
15. The method of claim 14, wherein the glomerular disease is a minimal change disease, a focal or segmental glomerulosclerosis, a collapsing glomerulopathy, a membranous
25 nephropathy, a membranoproliferative glomerulonephritis, a dense deposit disease, a cryoglobulinemia-associated glomerulonephritis, an IgA nephropathy, an Henoch-Schönlein disease, a postinfectious glomerulonephritis, a bacterial endocarditis, a pauci-immune crescentic glomerulonephritis, a Wegener granulomatosis, a

microscopic polyangitis, a Churg-Strauss syndrome, an anti-GBM-antibody mediated glomerulonephritis, a lupus nephritis, or a chronic allograft glomerulopathy.

16. The method of claim 14, wherein the glomerular disease has organized deposits.

17. The method of claim 16, wherein the glomerular disease is a amyloidosis, a
5 monoclonal immunoglobulin deposition disease, a fibrillary glomerulonephritis, or an immunotactoid glomerulopathy.

18. The method of claim 1, wherein the renal disorder is a tubulo-interstitial disease.

19. The method of claim 18, wherein the tubulo-interstitial disease is an ischemic tubular
10 injury, a medication-induced tubulo-interstitial nephritis, a toxic tubulo-interstitial nephritis, an infectious tubulo-interstitial nephritis, a bacterial pyelonephritis, a viral infectious tubulo-interstitial nephritis which results from a polyomavirus infection or an HIV infection, a metabolic-induced tubulo-interstitial disease, a mixed connective
15 disease, a cast nephropathy, a crystal nephropathy which may results from urate or oxalate or drug-induced crystals deposition, an acute cellular tubulo-interstitial allograft rejection, a tumoral infiltrative disease which result form a lymphoma or a post-transplant lymphoproliferative disease, or an obstructive disease of the kidney.

20. The method of claim 1, wherein the renal disorder is vascular disease.

21. The method of claim 20, wherein the vascular disease is a thrombotic
20 microangiopathy, a nephroangiosclerosis, an atheroembolic disease, a mixed connective tissue disease, a polyarteritis nodosa, a calcineurin-inhibitor induced-vascular disease, an acute cellular vascular allograft rejection, or an acute humoral allograft rejection.

22. The method of claim 1, wherein the renal disorder results from a hereditary or metabolic disease.

23. The method of claim 22, wherein the hereditary disease is Alport's syndrome, thin
25 membrane disease, Fabry's disease, a polycystic kidney disease, or a renal disorder which results from a metabolic disease is a diabetic nephropathy.

24. The method of claim 1, wherein the renal disorder is acute renal failure.

25. The method of claim 1, wherein the biomarker is present in the biological sample.
26. The method of claim 1, wherein the biomarker is absent from the biological sample.
27. The method of claim 1, wherein an increased amount of the biomarker in the biological sample relative to a control sample indicates that the individual has or is at risk of the renal disorder.
28. The method of claim 1, wherein the presence of the biomarker in the biological sample indicates that the individual has or is at risk of the renal disorder.
29. A method for identifying a candidate biomarker for a renal disorder comprising:
- a) obtaining a biological sample from an individual who has the renal disorder;
 - b) purifying a podocyte vesicle from the biological sample; and
 - c) measuring the amount of the protein in the biological sample;
- wherein if the protein is present in the biological sample in an amount greater than is present in a control sample, then the protein is the candidate biomarker.
30. The method of claim 29, wherein the candidate biomarker is greater than about 60 kDa.
31. The method of claim 29, wherein the candidate biomarker is a neuronal protein.
32. The method of claim 31, wherein the biological sample is a urine sample.
33. The method of claim 32, wherein the podocyte vesicle is extracted from the urine.
34. The method of claim 29, wherein the purifying comprises immunopurification.
35. A kit comprising:
- a) a first antibody preparation that selectively binds immunologically to of one or more biomarkers selected from the group consisting of the biomarkers of Table 1; and
 - (b) a suitable container means thereof.

36. The kit of claim 35, wherein the antibody selectively binds immunologically to pigment epithelium-derived factor, Attractin, FGF 11 (fibroblast growth factor), nestin, Dermcidin, EGF-containing fibulin-like matrix protein 1 (fibulin 3), myosin Va (non muscle), neuroligin-2, brain enriched hyaluronan binding protein, Ryanodine receptor 2, Ankyrin 3, serum paraoxonase/aryltransferase 1 (PON1), neuropilin-2 (NP-2), alpha2-macroglobulin or FERM domain containing protein 4A.
37. The kit of claim 36, wherein the antibody selectively binds immunologically to pigment epithelium-derived factor, FGF 11 (fibroblast growth factor), EGF-containing fibulin-like matrix protein 1 (fibulin 3), neuroligin-2, serum paraoxonase/aryltransferase 1 (PON1), neuropilin-2 (NP-2), or alpha2-macroglobulin.
38. The kit of claim 35, wherein said first antibody is a monoclonal antibody.
39. The kit of claim 35, wherein said first antibody is a polyclonal antibody.
40. The kit of claim 35, wherein said antibody preparation is attached to a support.
41. The kit of claim 40, wherein said support is a polystyrene plate, test tube or a dipstick.
42. The kit of claim 35, further comprising at least a second antibody preparation.
43. The kit of claim 42, wherein said second antibody preparation comprises a detectable label.
44. The kit of claim 43, wherein said detectable label is selected from the group consisting of a fluorescent tag, a chemiluminescent tag and an enzyme.
45. The kit of claim 44, wherein said enzyme is alkaline phosphatase or horseradish peroxidase.
46. The kit of claim 44, further comprising a substrate for said enzyme.
47. The kit of claim 35, further comprising a buffer or diluent and a suitable container means therefor.
48. The kit of claim 35, wherein the kit comprises an ELISA assay.